Apoptosis in Bovine Viral Diarrhea Virus (BVDV)- Induced mucosal disease lesions: A histological, immunohistological, and virological investigation

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Apoptosis in Bovine Viral Diarrhea Virus (BVDV)-induced mucosal disease lesions: A histological, immunohistological and virological investigation

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Running title

Mucosal disease and apoptosis

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Abstract

Cattle persistently infected with a non-cytopathic *Bovine viral diarrhea virus* (BVDV) are at risk of developing fatal "Mucosal Disease" (MD). We investigated the role of various apoptosis pathways in the pathogenesis of lesions in animals suffering from MD. Therefore, we compared the expression of caspase-3, caspase-8, caspase-9 and Bcl-2L1 (Bcl-x) in tissues of 6 BVDV-free control animals, 7 PI animals that showed no signs of MD (non-MD PI animals) and 11 animals with MD, and correlated the staining with the localization of mucosal lesions.

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This study clearly illustrates *ex vivo* that the activation of the intrinsic, but not the extrinsic, apoptosis pathway is a key element in the pathogenesis of MD lesions observed in cattle persistently infected with BVDV. However, whether direct induction of apoptosis in infected cells, or indirect effects induced by the virus are responsible for the lesions observed remains to be established.

***Key Words**:* Apoptosis; caspases; Bcl-x; bovine viral diarrhea virus (BVDV); cytopathic; noncytopathic; immunohistochemistry; Mucosal Disease

Introduction

Bovine viral diarrhea virus (BVDV) is an economically important animal pathogen that belongs to the genus pestivirus of the family *Flaviviridae*.[46] This virus exists as two biotypes, cytopathic (cp) and noncytopathic (ncp), according to the effect on cultured cells.[11,24,35] In pregnant cattle, ncp BVDV is able to cross the placenta, invade the fetus and cause persistent infection. After birth, persistently infected (PI) calves may develop fatal Mucosal Disease (MD). From animals succumbing to MD, both an ncp and a cp biotype can be isolated[4,7] that are antigenically very similar.[6,8,18] The cp BVDV evolves by mutation from the ncp biotype within PI animals (for reviews, see[26,32,34]).

Apoptosis is a tightly regulated physiologic and pathologic process of cell death that may be elicited by several molecular pathways. The most important ones are the extrinsic and intrinsic pathways. In the extrinsic pathway (also known as “death receptor pathway”), apoptosis is triggered by the ligand-induced activation of death receptors (e.g. Fas receptor, TNF receptor) at the cell surface, which leads to the activation of the initiator caspase-8. In the intrinsic pathway (also called “mitochondrial pathway”) apoptosis results from an intracellular cascade of events leading to the release of mitochondrial proteins followed by caspase-9 activation. Both the intrinsic and the extrinsic pathways finally converge in the activation of the executioners caspase-3 and caspase-7.[21,39] Among all these processes, anti-apoptotic members of Bcl-2 family, such as Bcl-2, Bcl-x_L and Bcl-w can provide protection against apoptosis that is mediated through the mitochondrial pathway.[22,45,49] This intrinsic pathway is suggested to be the most prominent cell death pathway in vertebrates,[21] and numerous viruses are known to cause

apoptosis of infected cells. However, apoptosis might have opposing effects on viral pathogenesis by either preventing viral dissemination due to death of infected cells or by enhancing virus spread.[27,33]

It has been previously reported that cell death associated with cp BVDV *in vitro* is mediated by apoptosis.[15,17,36,50] Infection of cell cultures with BVDV of the cp biotype is characterized by the predominant expression of the NS3 protein, the cleaved form of the non-structural protein NS2-3. In contrast, in cells infected with ncp BVD viruses the uncleaved NS2-3 precursor protein prevails.[23] It has been proposed that the NS3 protein of BVDV induces apoptosis that is correlated to caspase-8 and caspase-9 activation.[37] MDBK cells infected with cp BVDV showed an increase in caspase-3 and caspase-9 activity compared to cells infected with ncp BVDV. The level of caspase-3 and caspase-9 activity was dependent on the proteolytic activity of NS3/4A. On the other hand caspase-8 levels increased only slightly.[10] Furthermore, it has been demonstrated that macrophages infected with cp BVDV release a factor that promotes lipopolysaccharide (LPS)-induced apoptosis in uninfected macrophages by an interferon-independent pathway.[20,31] Similarly, it has been shown that TNF-α is released by primary bovine fetal muscle cells experimentally infected with cp BVDV and this might be involved in caspase-8 activation, albeit the effect was rather small compared to the strong stimulation of caspase-3 activity.[48] In other studies, oxidative stress,[36] endoplasmic reticulum (ER) stress,[19,27] intracellular viral RNA[42] or double-stranded (ds) RNA accumulation[47] and mitochondria dependent caspase-9 activation[13] have been reported to be associated with cp BVDV-induced apoptosis.

Recently, *in vitro* infection of a lymphoid cell line (BL3) indicates that not only the biotype but also the virulence of a given virus strain plays an important role in apoptosis induction. Thus, in contrast to infection with non virulent ncp BVDV, infection with a virulent ncp BVDV-2 induced disruption of the mitochondrial transmembrane potential similar to cp BVDV. However, the effect was less pronounced and delayed and a weaker activation of caspases was observed as compared to infection with a cp BVD type-2 virus.[3]

Thus, the majority of the *in vitro* studies point to an involvement of the intrinsic rather than the extrinsic apoptosis pathway in cell death caused by cp BVDV infection.[14,37,48] However, it is still unclear if this mechanism also participates in the development of lesions present in animals dying of MD and whether it is correlated with BVDV antigen expression *in vivo*. The aim of this retrospective study was, therefore, to investigate the role of the various apoptosis pathways in the pathogenesis of lesions in animals suffering from MD. For this purpose, we assessed and compared the expression of caspase-3, caspase-8, caspase-9 and Bcl-2L1 (Bcl-x) in tissues from BVDV free control animals, from PI animals that showed no signs of MD (non-MD PI animals) and in tissues from animals with MD as determined by the pathological findings.

Material and Methods

Animals

In this retrospective study, a total of 24 cattle, mostly female and of the Brown Swiss breed, were included. These animals were submitted to necropsy between

July 2000 and July 2007 to the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, with the diagnosis of BVDV infection. The cases were classified according to macroscopic changes, histological lesions, and immunohistochemical findings as non-MD PI (n = 7) or as MD cases (n = 11) (see supplementary material). In addition, virus isolation was done retrospectively, and only animals from which an ncp and a cp biotype of BVDV could be isolated where analyzed as MD cases (Table 1). The non-MD cases were classified according to immunohistochemical staining for viral antigen in the skin irrespective of virus isolation, as with this method, only persistently infected animals were indentified.[5,16,28] Sample tissues from 6 immunohistochemically BVDV negative animals were used as controls.

Immunohistochemistry

Immunohistochemical investigations were performed on paraffin-embedded sections from the following tissues: tongue, gingiva, esophagus, rumen, skin, lung, lymph node, spleen and ileum. Sections of two to three micrometers were mounted on positive-charged glass slides, deparaffinized, rehydrated through graded alcohol and washed in water. Slides for active caspase-3, caspase-9 and Bcl-2L1 (human Bcl-x) staining were twice pretreated by microwave cooking in a citric acid buffer (pH 6, DAKO®, Code No. S2031, DAKO, Zug, Switzerland) for 10 minutes at 750W and then counterstained with hemalaun for 2 minutes. Slides for caspase-8 and 15c5 staining were first counterstained with hemalaun and then pretreated with proteinase K (DAKO®, Code No. S2019) for 10 minutes at room temperature (RT).

After the corresponding pretreatment of the slides, endogenous peroxidases were inhibited by 3% H_2O_2 with 0.2% NaN_3 in water. Subsequently, a protein blocking step (DAKO®, Protein Block Serum-Free, Ready-to-Use, Code No. X0909) was performed. Both steps were carried out for 10 minutes at RT. Between the steps, the slides were carefully washed with PBS (pH 8).

After the pretreatment and blocking steps, the slides were incubated with the following antibodies at different dilutions over-night at RT: active caspase-3, dilution 1:200 (rabbit anti active capase-3, Code No. ab13847, Abcam, Cambridge, United Kingdom), Bcl-2L1, dilution 1:500 (rabbit anti BCL2L1 (human Bcl-x), Code No. ab45002, Abcam), 15c5, dilution 1:10.000 (mouse anti pestivirus gp48/E^{rns}, Dr. Dubovi, Cornell University, New York State, USA), caspase-8, prediluted (rabbit anti capase-8, Code No. ab15552, Abcam; this antibody is reported by the manufacturer to recognize both the full length caspase-8 and the processed forms) and caspase-9, prediluted (rabbit anti capase-9, Code No. ab13847, Abcam; as described by the manufacturer the immunogen used corresponds to the N-terminal amino acids 1-134 of human caspase-9). As secondary antibody, anti-rabbit EnVision™ (DAKO®, Code No. K4003,) or anti-mouse EnVision™ (DAKO®, Code No. K4001) was added for 30 minutes at RT. Subsequently, all slides were treated with AEC (3-amino-9-ethylcarbazole Substrate Kit, Code No. 00-2007, Invitrogen, Basel, Switzerland) for 10 to 15 minutes at RT. After controlling the color reaction and washing, the slides were mounted with an aqueous mounting solution (Kaiser's gelatin glycerin, HX631620, Fluka, Buchs, Switzerland).

For each of the immunohistochemical assays, a positive and a negative control were included. The following organs that displayed the highest reactivities were used as positive control: bovine thymus for active capase-3, bovine thymus and lymph node for Bcl-2L1 (human Bcl-x), bovine lymph node for caspase-8, bovine thymus for caspase-9 and brain tissue from a PI animal for 15c5 staining. As negative controls the same organs without primary antibody but incubated with PBS were used.

Biotype analysis

Organ samples of the thyroid gland or, when not available, the skin, tongue, or abomasum that had been stored at -20°C were used to determine the biotype of BVDV present in these samples. Approximately 30mg of frozen tissue was homogenized in 1.8 ml cell culture medium (Earle's minimal essential medium, MEM; Seromed (Biochrom) Munich, Germany) enriched with 2% fetal bovine serum (FBS; Sigma or Oxoid GmbH, Wesel, Germany) using the Qiagen Tissue Lyser (Qiagen AG, Hombrechtikon, Switzerland). The homogenate was cleared by centrifugation at 13'000 g prior to diluting it seven times in ten-fold steps in MEM. FBS was free of BVDV and antibody to BVDV as tested by virus isolation and serum neutralization test, respectively. Each dilution step was distributed to six wells (100µl per well) of a 96-well microtiter plate, seeded with primary bovine turbinate cells prepared as described.[36] After five days of incubation at 37°C and 5% CO_2, 20µl of supernatant was transferred to a fresh 96-well microtiter plate, pre-seeded with bovine turbinate cells. After addition of 80µl of fresh MEM, the

microtiter plate was incubated as before and the passaging procedure of the supernatant was repeated once more. After each passage, the cells were fixed and stained for viral protein by immunoperoxidase staining using a polyclonal swine-α-BVDV antibody produced at the Institute of Veterinary Virology, University of Bern. In addition, the cells were microscopically examined for the presence of a cytopathic effect.

BVD virus biotype, genotype and subgroup determination

Viral RNA was isolated from frozen organ samples as used for the biotype analysis. Prior to RNA isolation, approximately 30mg of frozen tissue was homogenized in RLT buffer (Qiagen) using the Qiagen Tissue Lyser and the homogenates were cleared by centrifugation. The RNeasy mini and viral RNA kits from Qiagen were used following the manufacturer's instructions for RNA isolations from tissues and sera, respectively. All RT-PCR reactions were done as one-step and single tube reactions using the OneStep RT-PCR kit (Qiagen). For amplification of the 5'UTR the pan-pesti primer pair 324/326 was used,[43] yielding a DNA fragment of 288 nucleotides based on the reference strain NADL. PCR products to be sequenced were separated by 1% agarose gel electrophoresis and DNA fragments of the correct size were isolated using the QIAquick gel extraction kit (Qiagen). After spectrophotometrical quantification of the DNA at 260nm, the required amount of DNA, primer and water (aqua ad injectabilia, Dr. G. Bichsel AG, Interlaken, Switzerland) was mixed and sent to Microsynth GmbH (Balgach, Switzerland) for sequencing. The economy run and premixed primer options were used. The sequencing data were assembled and aligned using the SeqMan

(DNASTAR Inc., Madison, USA) and Clone Manager (Scientific & Educational software, Cary, USA) software, respectively. Phylogenetic analysis was performed using the programs included in the GCG software package as PILEUP for the multiple sequence alignment and PAUP for calculating the distances by the Kimura 2-parameter method. To assess the BVDV genotype and subgroup, phylogenetic trees were constructed according to the neighbor-joining method including 1000 bootstrap replicates. The genetic classification (Table 1) was performed for BVDV genotype 1 as described.[44]

Data analysis

The distribution of morphologic tissue lesions was compared blindly, i.e. not knowing the BVDV status of the sample, to the immunohistochemical detection of active caspase-3, caspase-9, caspase-8, Bcl-x (Bcl-2L1) and the BVD viral protein E^{rns}. The intensity of the staining was analyzed by optic microscopy, the number of positive cells were counted in 10 random fields (magnification: 20x) and the average of the 10 high power fields were scored accordingly to the following scale: (-) = no immunohistochemical staining; (+) = single positive cells; + = slight positive staining, up to 15% positive cells; ++ = moderate positive staining, from 15 to 30% positive cells, and +++ = strong positive staining with more than 30% positive cells (Table 2).

Results

BVDV antigen expression

Cells that were positively labeled with BVDV/MD immunohistochemistry were mostly epithelial cells, including basal cells as well as hair root sheath epithelial cells in the skin, and most layers of the squamous epithelium of the tongue, esophagus and rumen. In the ileum only few epithelial cells in the crypts were positive. Also positive were fibrocytes, endothelial and medial cells of blood vessels, few lymphocytes in various lymphatic tissues and occasionally lung macrophages. In the brain that was used as positive control, neurons, endothelial cells and some glial cells were positive.

All MD and non-MD PI cases displayed positive immunohistochemical staining with the 15c5c antibody directed against the viral glycoprotein E^{rns}. Most layers of the epithelial cells in non-MD PI cases showed a strong intracytoplasmic labeling with the 15c5 antibody. No difference was visible between the staining intensity in tissue without lesions in both MD and non-MD PI groups. In areas of mucosal lesions the staining was diminished due to loss and degeneration of epithelial cells. Control animals were negative with the same antibody (Figs. 1-3, panels b; Table 2).

Expression of active caspase-3

All animals with MD displayed a positive intracytoplasmic staining for active caspase-3 in all tissues analyzed (Fig. 2c; Table 2). In tongue, gingiva, esophagus, rumen and ileum the positive caspase-3 staining was correlated to areas of

lesions, such as erosions or ulcerations in the tongue and depletion of Peyer´s patches. Positive staining was mainly seen in epithelial cells of the basal layer in and adjacent to the eroded and ulcerated areas. In addition, positive staining was observed in neutrophils, fibrocytes and crypt epithelial cells in the ileum and in lymphocytes of the Peyer's patches.

In contrast to the MD cases, the non-MD PI animals showed no or very discrete and localized caspase-3 staining in tongue (mostly tongue papillae), gingiva, esophagus and rumen. In lymphoid tissues (BALT, lymph node, spleen, ileum), however, the expression of caspase-3 was similar as compared to the MD cases. In the skin, sebaceous glands and hair follicles showed moderate positive staining in all groups (non-MD, MD and controls).

The negative control animals showed only slight caspase-3 staining in mucosal tissues, and only a few positive cells were detected in lymphoid tissues like spleen, lymph node and ileum.

Expression of caspase-8

The expression of this protein was mainly detected in sebaceous glands and hair follicles of the skin of all three groups of animals. In the Peyer's patches only few positive lymphocytes were visible in the MD cases. All other organs were negative or very weakly positive (e.g. single positive epithelial cells) (Figs. 1-3, panels d; Table 2).

Expression of caspase-9

The expression pattern of caspase-9 in MD cases was very similar to the results found with the active capase-3 antibody (Table 2). All samples analyzed from animals with MD displayed a positive staining in tongue, gingiva, esophagus, rumen and ileum. In addition, caspase-9 positive labeling was correlated to areas of erosion and ulceration in these organs and to the depletion of Peyer´s patches (Fig 2e). In lymph nodes and spleen a few lymphocytes in the follicle area and in the lung in the BALT region were positive.

Non-MD PI animals showed discrete staining in tongue, gingiva, ruminal epithelium (Fig. 1e), and esophagus. The expression of caspase-9 in lymphoid tissues and lung was similar to the staining in the MD cases.

Negative control animals displayed a weak to moderate positive labeling in the epithelium of the digestive tract organs analyzed and in organs like lymph node, spleen and lung. The staining was similar or even stronger than seen in the non-MD PI animals (Fig. 3e; Table 2). Thus, it was not possible to distinguish the intensity of expression of caspase-9 between non-MD, MD and control groups in the lymph node, spleen and lung. However, the intensity of staining of epithelial cells was stronger in the MD cases than in the non-MD and control animals and correlated with ulcerative or erosive lesions.

Expression of Bcl-x (Bcl-2L1)

In MD cases, the expression of Bcl-x was detected in areas of erosions and ulcerations of tongue, gingiva, and esophagus and in areas of depletion of the Peyer´s patches (Table 2). The cells showing strong positive staining were primarily epithelial cells from the upper layers of the stratified squamous epithelium

and weaker staining was visible in the basal cell layers (Fig. 2f). In addition, some inflammatory cells like neutrophils and lymphocytes of the Peyer's patches were positive.

In non-MD PI animals, Bcl-x expression was also observed, although weaker and mainly in the upper epithelial cell layers but in the same tissues as described for the MD cases (Fig. 1f). Weak Bcl-x staining was also observed in other tissues like in the follicles of the lymph node, in few lymphocytes in the periarteriolar sheaths of the spleen and in some lymphocytes of the BALT in the lung from all animals studied. Generally, the staining pattern was overall stronger in MD cases than in non-MD PI and BVD negative cases (Figs. 1-3, panels f; Table 2).

Biotype and subgroup analysis

BVD viruses of the cp biotype in addition to the persisting ncp strain could be isolated from 11 animals that were therefore classified as MD cases (Table 1). As expected, no cp biotype was isolated from the animals of the non-MD group.

The viruses present in the MD and non-MD PI animals belonged to the subgroups 1h (7), 1k (3), 1e (3), 1b (1) and 1l (1). From 3 animals the subgroup identification was not done (Table 1). The spectrum of subgroups was similar in the MD and the non-MD PI animals and reflects that of BVD-1 viruses described to circulate in Switzerland.[1,38]

Discussion

In this retrospective study we investigated the apoptotic pathways, extrinsic or intrinsic, implicated in BVDV persistently infected animals, either suffering from MD

or showing no macroscopical or histological signs thereof. The immunohistochemical staining used in this study is a highly specific and sensitive method to detect apoptotic cells in archival paraffin-embedded material.[12] Due to the limited availability of the study material, further analyses, e.g. using western blotting or real-time RT-PCR, could not be applied. However, this might not be disadvantageous as the latter methods are bulk cell measurements that are inherently less informative than immunohistochemistry.

Thus, by means of immunohistochemistry, MD cases showed more caspase-3 and slightly more caspase-9 expression than non-MD PI animals. Interestingly, the caspase-3 and -9 staining was correlated to erosive and ulcerative lesions in organs of the digestive tract (tongue, gingival, esophagus, rumen and ileum) while this was not the case in the other organs analyzed (skin, lung, lymph node and spleen). This phenomenon might be correlated to the presence of bacteria or bacterial constituents such as LPS in the digestive tract. As previously shown, cp BVD virus primes uninfected macrophages for LPS-induced apoptosis,[20,31] which might additionally contribute to the cell death observed in the gastrointestinal tract. However, in contrast to caspase-3 staining that was markedly stronger in BVDV infected animals, caspase-9 expression was also quite high in the negative control animals.

These results suggest that caspase-3 and -9 and, thus, the intrinsic apoptotic pathway is involved in the pathogenesis of the mucosal lesions typically observed in MD cases. This is corroborated by the fact that caspase-8 was not elevated in our study, leading to the conclusion that the extrinsic pathway is rather not involved. However, it remains to be clarified whether the intrinsic pathway is

directly or indirectly induced by the BVD virus or rather by unspecific activation promoted by the tissue damage. In a recent study of fetal and neonatal small ruminants naturally infected with Border Disease Virus (BDV), neuropathological analysis of the central nervous system indicated that the intrinsic pathway of apoptosis was most important, but that both the intrinsic and extrinsic pathways are involved in infection with a non-cytopathic BDV.[41] Whether the participation of the extrinsic pathway is restricted to certain pestiviruses or to different cell types is, however, not yet known.

Recently, experimental acute infection of calves with an ncp biotype of BVDV 1 led to a progressive depletion of the Peyer's patches. The depletion was due to massive lymphocyte apoptosis with concurrent activation of caspase-3 as analyzed by immunohistochemistry.[30] However, lymphocyte apoptosis in the gut-associated lymphoid tissue (GALT) was paralleled by an increase in caspase-8 and rather a decrease in caspase-9, indicating that the extrinsic rather than the intrinsic pathway of apoptosis might predominate in acute BVDV infections.[29]

This in contrast to the results presented in this study that showed that the intrinsic pathway is a key element in the induction of apoptosis in MD pathogenesis in persistently infected calves. Therefore, cell death induced by a cp biotype of BVDV in an immunotolerant animal seems to occur through different pathways than during acute infections with ncp BVD viruses. Moreover, changes in cell proliferation or immune-mediated cell damage induced by cp BVDV might additionally contribute to the mucosal atrophy observed in MD.[9,25] Thus, it is rather probable that direct virus-induced cell death as well as indirect mechanisms contribute to the overall pathology observed in MD.

Interestingly, not only the expression of caspases-3 and -9 but also the protein Bcl-x co-localized with the mucosal lesions of the MD cases. It was shown in one study *in vitro* that induction of cellular apoptosis by a cp strain of BVDV-1 correlates with down-regulation of the anti-apoptotic Bcl-2 protein. However, at early time points post-infection, there was rather an increase in Bcl-2 expression.[19] In another study, the Bcl-2 protein was up-regulated by ncp BVDV infection, presumably suppressing the activation of executioner caspases and the initiation of cell death, thus enabling persistent infection *in vitro*.[2] In our study, Bcl-x had slightly stronger expression in tissues from MD cases than in non-MD PI and non-infected control animals. Thus, it remains to be investigated whether Bcl-x, its splice variant Bcl-x_S[40] or other members of the Bcl-2 family have a role in inhibition of apoptosis or even in activation of cell death; and whether they are directly modulated by BVDV or rather in an unspecific way.

The viral subgroups present in the samples analyzed encompass the complete spectrum of strains circulating in Switzerland.[1] There was no correlation of a certain genetic subgroup to a specific type of macroscopic or immunohistochemical finding. This makes strain-specific differences in apoptosis very unlikely, despite that only a small number of animals per subgroup could be analyzed.

In conclusion, markers of the intrinsic apoptotic pathway were activated in mucosal lesions of animals suffering from MD, which corresponds to the *in vitro* findings that activation of the initiator caspase-9 and the executioner caspase-3 might play a major role in cp BVDV induced cell death. However, we were unable to demonstrate that cells with enhanced staining for activated caspase-3 were directly infected with cp BVDV. The fact that strong caspase-3 and -9 staining was

predominantly detected in lesions of the gastrointestinal tract, i.e. in regions rich in LPS or other bacterial constituents, might indicate that priming of uninfected cells by cp BVDV for activation-induced apoptosis additionally contributes to the formation of the lesions observed. Thus, further *in vivo* studies are required to unravel the complex pathogenesis of the clinical and necropsy findings observed in this lethal disease.

Figure legend:

Figs. 1a to 3a: Bovine, rumen, HE-staining.

Fig. 1a: Non-MD PI animal, no histological lesions are visible.

Fig. 2a: MD case, the epithelial cells are detached from the lamina propria and show vacuolar degeneration.

Fig. 3a: Control animal, no histological changes can be seen.

Figs. 1b to 3b: Bovine, rumen, BVDV immunohistochemistry, hemalaun counterstain.

Fig. 1b: Non-MD PI animal, most of the epithelial cells and some fibrocytes show strong homogenous intracytoplasmic staining.

Fig. 2b: MD case, only few of the detaching epithelial cells show positive intracytoplasmic labeling.

Fig. 3b: Control animal, no labeling is visible.

Figs. 1c to 3c: Bovine, rumen, caspase-3 immunohistochemistry, hemalaun counterstain.

Fig. 1c: Non-MD PI animal, no labeling is present.

Fig. 2c: MD case, most of the detached epithelial cells show strong intracytoplasmic staining.

Fig. 3c: Control animal, no labeling is visible.

Figs. 1d to 3d: Bovine, rumen, caspase-8 immunohistochemistry, hemalaun counterstain.

Fig. 1d: Non-MD PI animal, no labeling is present.

Fig. 2d: MD case, no labeling is visible.

Fig. 3d: Control animal, no labeling is present.

Figs. 1e to 3e: Bovine, rumen, caspase-9 immunohistochemistry, hemalaun counterstain.

Fig. 1e: Non-MD PI animal, only some of the basally located epithelial cells show a intracytoplasmic staining.

Fig. 2e: MD case, labeling is strong in the detaching epithelial cells.

Fig. 3e: Control animal, upper epithelial cells are moderately stained, basally located are strongly labeled.

Figs. 1f to 3f: Bovine, rumen, Bcl-x immunohistochemistry, hemalaun counterstain.

Fig. 1f: Non-MD PI animal, Bcl-x staining shows a distinct homogenous intracytoplasmic staining of the superficial epithelial cells.

Fig. 2f: MD case, detached epithelial cells show a strong homogenous staining.

Fig. 3f: Control animal, superficial epithelial cells are strongly labeled.

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Table 1: Identification of BVDV subgroups and biotypes

MD		
Cases	***Subgroup***	***Biotype***
1	1b	ncp + cp
2	1e	ncp + cp
3	1h	ncp + cp
4	1h	ncp + cp
5	1h	ncp + cp
6	1h	ncp + cp
7	1h	ncp + cp
8	1k	ncp + cp
9	1k	ncp + cp
10	1l	ncp + cp
11	n.d.	ncp + cp

Non-MD PI		
Cases	***Subgroup***	***Biotype***
1	1e	ncp
2	1h	ncp
3	1h	ncp
4	1k	Isolation negative
5	1e	n.d.
6	n.d.	n.d.
7	n.d.	n.d.

MD: mucosal disease; non-MD PI: persistently infected animals; n.d.: not done.

Table 2: Immunohistochemical analysis

Antibody	Organ	MD-cases (n=11)		Non-MD PI animals (n=7)		Controls (n=6)	
		Number[a]	Staining[b]	Number[a]	Staining[b]	Number[a]	Staining[b]
BVDV-E^{rns}	Tongue	5	++	2	+to++	3	–
	Gingiva	3	+to++	1	+	2	–
	Esophagus	4	++to+++	1	+	6	–
	Rumen	3	+to++	3	+to++	1	–
	Skin	2	++	2	+to++	3	–
	Lung	6	+to++	3	+to++	3	–
	Lymph node	3	+to++	2	+to++	3	–
	Spleen	8	+	5	+to++	3	–
	Ileum/P.p[c]	6	+to++	3	+	3	–
Caspase-3	Tongue	5	***++***	2	+ [d]	3	+ [d]
	Gingiva	3	***+to++***	1	–	2	–
	Esophagus	4	***++***	1	+	6	+
	Rumen	3	***++to+++***	3	–to(+)	1	+
	Skin	2	+	2		3	+
	Lung	6	+to++ [e]	3	+to++ [e]	3	+ [e]
	Lymph node	3	+to++	2	+to++	3	+to++
	Spleen	8	+to++	5	+	3	+
	Ileum/P.p[c]	6	***+to++***	3	+to++	3	+to++
Caspase-8	Tongue	5	(+)	2	–	3	–
	Gingiva	3	–	1	–	2	–
	Esophagus	4	(+)	1	–	6	–
	Rumen	3	–	3	–	1	–
	Skin	2	+	2	+	3	+
	Lung	6	–	3	–	3	–
	Lymph node	3	(+)	2	(+)	3	(+)
	Spleen	8	(+)	5	(+)	3	(+)
	Ileum/P.p[c]	6	+	3	–	3	–
Caspase-9	Tongue	5	***++***	2	+	3	+to++
	Gingiva	3	***+to++***	1	+	2	+
	Esophagus	4	***+to++***	1	+	6	+to++
	Rumen	3	***++***	3	++	1	+
	Skin	2	+	2	+	3	+
	Lung	6	+to++ [e]	3	+to++ [e]	3	+to++ [e]
	Lymph node	3	++	2	++	3	+to++
	Spleen	8	++	5	++	3	+to++
	Ileum/P.p[c]	6	***+to++***	3	+to++	3	+to ++
BCL2L1	Tongue	5	***+++***	2	++	3	++
	Gingiva	3	***++***	1	++	2	++
	Esophagus	4	***++to+++***	1	+to++	6	+to++
	Rumen	3	+	3	++	1	+to++
	Skin	2	++	2	++	3	+
	Lung	6	+to++	3	+to++	3	+to++
	Lymph node	3	+to++	2	+to++	3	+to++
	Spleen	8	+to++	5	+to++	3	+to++
	Ileum/P.p[c]	6	***+to++***	3	+to++	3	+

Legend:
Cases where a co-localization of positive cells and of lesions was observed are highlighted in ***bold*** & *italics*.

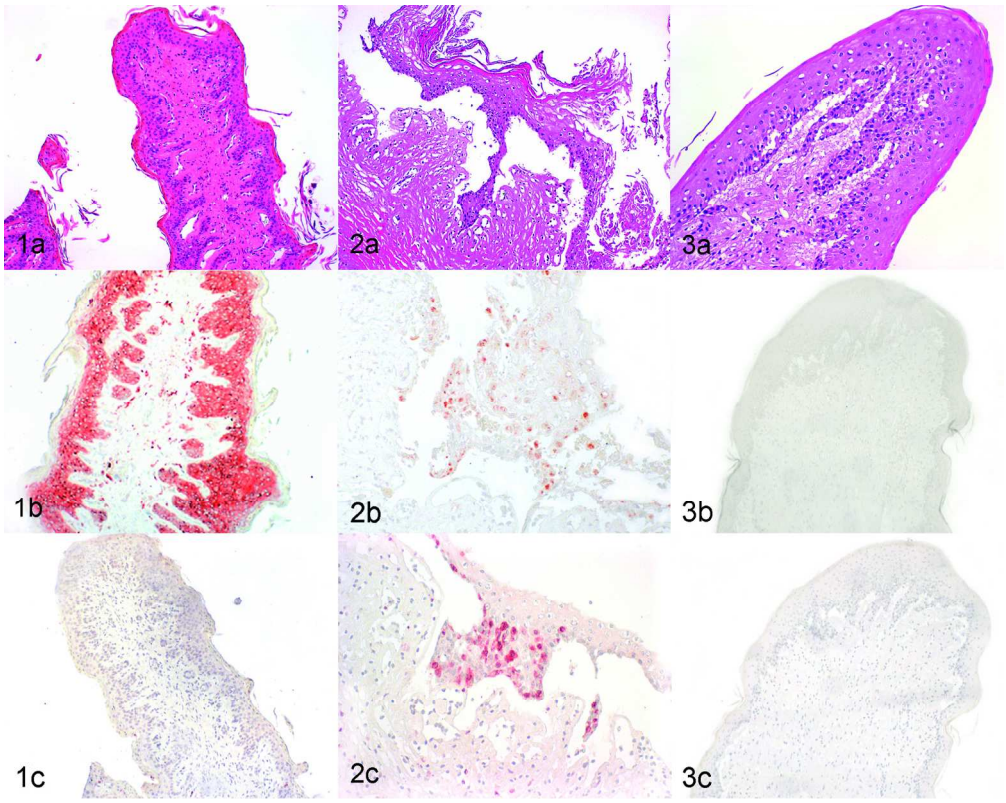
[a] Number of cases, where the respective tissue was available for analysis.

[b] Values of the cases analyzed. –: negative; (+): single positive cells; +: up to 15% positive cells; ++: 15–30% positive cells; +++: more than 30% positive cells.

[c] P.p: Peyer's patches.

[d] Tongue: only positive reaction in the papilla.

[e] Lung: most positive cells in BALT.



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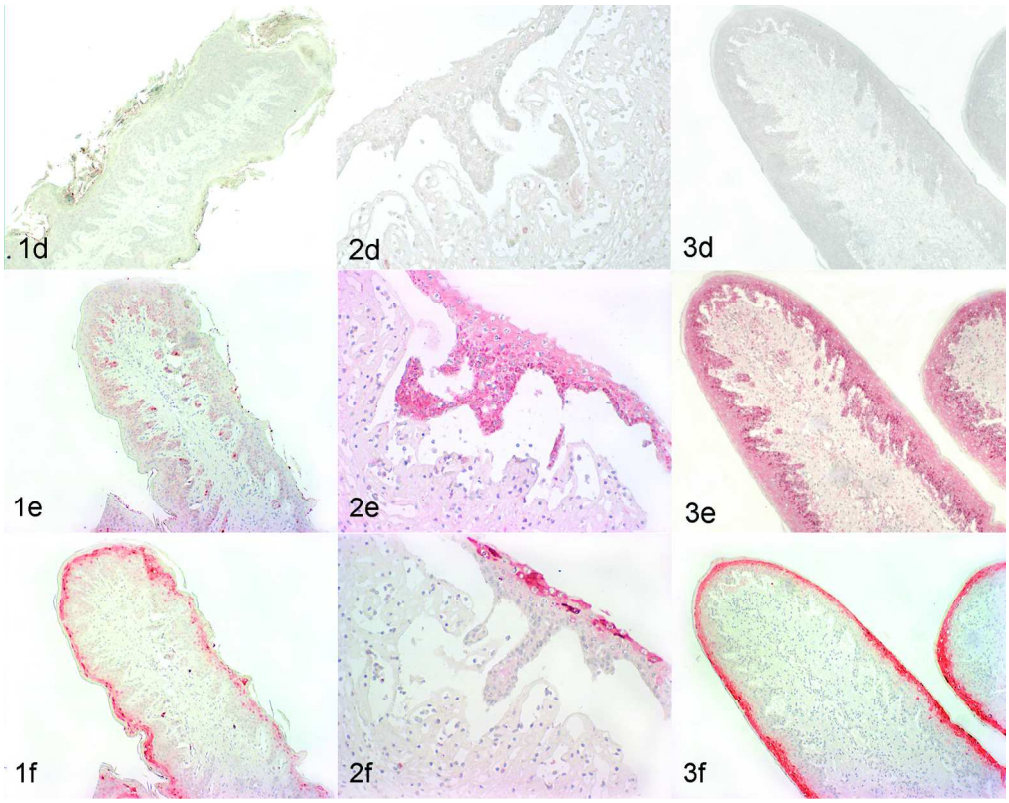

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